- 1 -

17. März 2000
Bundesrepublik Deutschland
V29698PCUS BÖ/El/ps

ANTI- HUMAN T-CELL COSTIMULATING POLYPEPTIDS MONOCLONAL ANTIBODIES

~~Costimulating polypeptide of T cells, monoclonal anti-~~
~~bodies, and the preparation and use thereof~~

The invention relates to a polypeptide (8F4
molecule) having the biological activity of costimula-
ting T cells. The invention further relates to monoclo-
nal antibodies against the 8F4 molecule and hybridoma
cells which produce the monoclonal antibodies. The in-
vention additionally relates to the use of substances
which inhibit the biological activity of the polypepti-
de 8F4 according to the invention, in particular mono-
clonal antibodies, natural or synthetic ligands, ago-
nists or antagonists, as pharmaceuticals. In particu-
lar, the invention relates to the use of these substan-
ces for the prevention or therapy of disorders in which
the immune system is involved, in particular for the
treatment of autoimmune diseases and for the prevention
of rejection reactions with organ transplants. The in-
vention additionally relates to the use of the 8F4 mo-
lecule or of cells which contain the 8F4 molecule as
pharmaceuticals, in particular for the prevention or
therapy of disorders in which the immune system is in-
volved, in particular for the treatment of cancers,
Aids, asthmatic disorders or chronic viral diseases
such as HCV or HBV infections. The invention further
relates to the use of substances which specifically re-
cognize the polypeptide according to the invention, in
particular monoclonal antibodies, natural or synthetic
ligands, agonists or antagonists, for the diagnosis of
disorders in which the immune system is involved. In
particular, the invention relates to diagnosis by means
of an ELISA detection, a flow cytometry or a Western
blot, a radioimmunological detection, a nephelometry or
a histochemical staining.

T lymphocytes recognize their antigen, which
is presented by "antigen-presenting cells", for example
dendritic cells, B cells and macrophages, through their
T-cell receptor. Recognition of the antigen by the
T-cell receptor alone is, however, in most cases insuf-
ficient for adequate activation of T lymphocytes. The
latter makes additional simultaneous stimulation (also
called "costimulation" hereinafter) by other receptor
molecules on the surface of the T lymphocytes necessa-

ry. One of these receptor molecules is the so-called
CD28 receptor which is stimulated by the costimulating
molecule B7. If these "costimulatory" molecules, for
example CD28, are effective, then the activation of the
5   T cell reaches an adequate level after recognition of
the antigen by the T-cell receptor. After such a com-
plete activation, the T cell expresses additional mole-
cules, for example CD25, CD69, CD71, on the surface and
synthesizes numerous cytokines, for example IL-2 and
10   IFN-γ, which function as messengers. Both these additio-
nal surface molecules and the cytokines serve for the T
cell to exchange information with other cells in the
immune system. The activated T cells direct the entire
antigen-specific immune defences through the additional
15   surface molecules and the cytokines. Both the generati-
on of cytotoxic cells ("killer cells") and the genera-
tion of antigen-specific antibodies by B cells is con-
trolled in this way. Cytotoxic cells, as well as the
specifically formed antibodies, eliminate viral or bac-
20   terial pathogens which enter the body. In some cases,
however, the immune response goes too far, and the im-
mune system is directed against the body's own cells.
This leads to the occurrence of "autoimmune diseases",
for example to rheumatoid arthritis, ankylosing spondy-
25   litis, Sjögren's syndrome, ulcerative colitis inter
alia. One of the essential sites of cooperation between
antigen-activated T cells and other cells of the immune
system are the secondary lymphatic organs, including
the tonsils. This is where the T lymphocytes are acti-
30   vated by the antigen presented by dendritic cells, and
this is where T lymphocytes interact with B cells.
Through this interaction, B cells secrete, after se-
veral intermediate stages of differentiation, antigen-
specific antibodies of the IgM and IgG types.

35   The costimulatory molecule which has been
characterized best and is among the most effective to
date is the CD28 surface molecule (called CD28 receptor
or CD28 hereinafter) which is constitutively expressed
on a large fraction of T cells. Costimulation by CD28
40   *in vitro* leads, after recognition of the antigen by the
T-cell receptor, to a very large increase in cytokine
secretion, for example of IL-2 and IFN-γ, and to a mar-
ked up-regulation of the expression of cell surface mo-
lecules such as CD25, CD69, CD71, which are necessary
45   for interaction of T cells with other immune cells, for
example B lymphocytes; cf. Chambers and Allison, *Cur-
rent Opinion in Immunology* 9 (1997), 396-404. Costimu-
lation via the CD28 receptor can also markedly increase
the proliferation of T lymphocytes. In addition, costi-

mulation via the CD28 receptor optimizes the T-cell control of B-lymphocyte function so that there is increased secretion of antibodies.

5   If the function of the CD28 receptor is abolished, there is a drastic loss of function in the immune defences. This has been shown by means of a transgenic mouse in which the CD28 gene was destroyed by homologous recombination (a so-called "CD28 knock-out").
The destruction in this way of activation of the anti-
10   gen-specific T cells leads to lack of costimulation. This in turn leads to a disturbance of T-cell function, that is to say to a reduced proliferation of T cells and to a drastically reduced synthesis of various cytokines. The lack of costimulation eventually leads to a
15   reduced function of the antigen-specific immune defences. Thus, inter alia, the formation of antigen-specific IgG1 and IgG2 antibodies by B lymphocytes is reduced to 10% of the normal level through the lack of CD28; cf. Shahinian et al., *Science* 262 (1993), 609-
20   612; Lucas et al. Journal of *Immunology* 154 (1995), 5757-5768. It is also possible *in vitro* to prevent the Aids virus entering T lymphocytes by costimulation by CD28; cf. Riley et al., *Journal of Immunology* 158 (1997), 5545-5553. Corresponding experiments *in vivo*
25   have not yet been carried out. It is known that CD28 switches on many cytokine genes which may lead to considerable side effects *in vivo*. Blockade of CD28 receptors by a soluble CTLA-4 immunoglobulin molecule has been employed successfully in a monkey model to prevent
30   the rejection of transplanted kidneys. In this case, CTLA-4 had been employed in combination with an antibody against the CD40 ligand molecule; cf. Kirk et al., *Proc. Natl. Acad. Sci.* USA 94 (1997) 8789-8794. However, blockade of CD28 receptors affects all T lym-
35   phocytes and not just those already activated because CD28 is constitutively expressed on T lymphocytes.

There is thus a need for a costimulating surface molecule which is expressed only on activated T lymphocytes. The invention is therefore based on the
40   object of providing a surface molecule on activated T cells which has a strong costimulatory effect on central functions of T lymphocytes. Another object of the invention is to provide substances, for example monoclonal antibodies against the costimulatory surface mo-
45   lecule, natural or synthetic ligands, agonists or antagonists of the surface molecule.

In a first embodiment, the invention relates to a polypeptide having the biological activity of co-

stimulation of T cells, characterized in that a) the polypeptide occurs on activated CD4$^+$ and CD8$^+$ T lymphocytes but not on resting or activated B cells, granulocytes, monocytes, NK cells (natural killer cells)

5 or dendritic cells, and b) the polypeptide is a dimer, the polypeptide having a molecular weight of about 55 to 60 kDa (kilodalton) determined in a non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the two polypeptide chains of the

10 polypeptide having a molecular weight of about 27 kDa and about 29 kDa measured in a reducing SDS-PAGE.

The polypeptide according to the invention (also called 8F4 molecule or 8F4 hereinafter) is expressed only after activation of the T lymphocytes,

15 specifically both on CD4$^+$ and on CD8$^+$ T cells. In a non-reducing SDS-PAGE, the 8F4 molecule has a molecular weight between about 55 and 60 kDa (kilodalton). The 8F4 molecule is composed of two peptide chains, and the two peptide chains have a molecular weight of about 27

20 and about 29 kDa in a reducing SDS-PAGE. The 8F4 antigen can be unambiguously detected histologically on activated T lymphocytes in the lymphatic tissue of the tonsils and lymph nodes, especially in the germinal centres, the site of interaction of T lymphocytes and B

25 lymphocytes in the generation of antibodies. Tonsillar T cells isolated *ex vivo* are about 50-80% positive for the 8F4 antigen and show signs of advanced activation. The 8F4 molecule is not detectable on resting or activated B cells, granulocytes, monocytes, NK cells and

30 dendritic cells.

An important biological activity of the 8F4 molecule is its costimulating activity on T lymphocytes. The costimulating activity can be determined by the method of Linsley et al., *Journal of Experimen-*

35 *tal Medicine* 176 (1992), 1595-604. The costimulating activity of the 8F4 molecule resembles the costimulating activity of the CD28 molecule, which has been identified as the central enhancement element of antigen recognition by the immune system. The 8F4 molecule

40 differs in many aspects from CD28, however. Thus, expression of the 8F4 molecule on the surface of the T cells requires induction, whereas CD28 is constitutively expressed. There are also distinct differences detectable in the function: costimulation by CD28 leads

45 to overexpression of numerous lymphokines, inter alia of interleukin-2 (IL-2). Costimulation by 8F4 also leads to enhanced secretion of lymphokines, but not of IL-2. The costimulatory activity of the 8F4 molecule thus differs from the activity of the CD28 molecule.

Since stimulation by 8F4 does not switch on all cytokine genes, costimulation by 8F4 *in vivo* is advantageous, for example compared with costimulation via the CD28 receptor. Moreover, the induction, the expression, the
5   site of expression and the function of the 8F4 molecule differ from all other known molecules with costimulatory activity.

The 8F4 molecule according to the invention is a novel surface molecule on activated T cells which
10   has a strong costimulatory effect on central functions of T lymphocytes. Expression *in vivo* indicates inter alia an essential involvement of the 8F4 molecule in the cooperation of T cells with other cells of the immune system such as B cells or dendritic cells within
15   the humoral and cellular immune defences against viruses and bacteria.

After expression, the 8F4 molecule has *in vitro* a strong costimulatory effect on various functions of T lymphocytes:

20   1.   Marked enhancement of the proliferation of T lymphocytes.

2.   Marked enhancement of the synthesis of certain cytokines by T lymphocytes.

3.   Greatly increased expression of control molecules,
25   for example surface molecules and cytokines, on and in T lymphocytes.

4.   Marked improvement in T-cell-induced antibody formation (IgM and IgG) by B cells.

The present invention furthermore provides a polypepti
30   de having the biological activity of costimulation of T cells and having an amino acid sequence which shows at least 40% homology with the sequence comprising 199 amino acids in Fig. 15 (SEQ ID NO:2), or a biologically active fragment or an analogue thereof. A biolo
35   gically active fragment or analogue is a fragment or analogue which likewise shows a costimulatory effect on T-cell lymphocytes or at least displays a biological effect of the nature of a blockage. Preference is given to a polypeptide or a biologically active fragment or
40   analogue thereof which shows at least 60% homology with the sequence comprising 199 amino acids in Fig. 15 (SEQ ID NO:2). In a particularly preferred embodiment, the polypeptide according to the invention comprises an amino acid sequence which shows at least 80% homology
45   with the sequence comprising 199 amino acids in Fig. 15

(SEQ ID NO:2), or a biologically active fragment or analogue thereof.

A particularly preferred polypeptide has the biological activity of costimulation of T cells and comprises an amino acid sequence as shown in Fig. 15 (SEQ ID NO:2), or a biologically active fragment or an analogue thereof.

The invention includes allelic variants, fragments and analogues of the 8F4 molecule. These variants include naturally occurring allelic variants, substitution analogues in which one or more amino acids have been substituted by different amino acids, substitution analogues in which one or more amino acids have been substituted by different amino acids, deletion analogues in which one or more amino acids have been deleted and addition analogues in which one or more amino acids have been added. Deletion and addition of one or more amino acids may be done either at an internal region of the polypeptide or at the amino or carboxyl terminus.

Polypeptides according to the invention fused to heterologous polypeptides are likewise embraced.

In another embodiment, the invention relates to DNA sequences which encode a polypeptide according to the invention or a biologically active fragment or analogue thereof.

These DNA sequences include the sequence shown in SEQ ID NO:1 (Fig. 16) as well as allelic variants, fragments, and analogues having biological activity.

A preferred DNA sequence encodes a polypeptide having the biological activity of costimulation of T cells, the sequence being selected from the group consisting of:

a) the DNA sequence shown in SEQ ID NO:1 (Fig. 16) and its complementary strand

b) DNA sequence hybridizing with the sequences in (a) and

c) DNA sequences which, because of the degeneracy of the genetic code, hybridize with the sequences in (a) and (b). The aforementioned DNA sequences preferably hybridize together under stringent conditions.

Also provided are vectors which comprise these DNA sequences, and host cells which are transformed or transfected with these vectors.

In another embodiment, the invention relates to monoclonal antibodies against the 8F4 molecule. The monoclonal antibodies according to the invention can be prepared in a conventional way by the method described by Milstein and Köhler, *Nature* 256 (1975), 495-497. In particular, the monoclonal antibodies according to the invention can be prepared by immunizing mice with T cells which have been activated *in vitro* with phorbol myristate acetate (PMA) and ionomycin ("2-signal system") for 24 h. The spleen cells of the immunized mice are fused with myeloma cells. 8F4-specific monoclonal antibodies are identified by their recognition of 2-signal-activated but not resting T lymphocytes. Moreover 8F4-specific antibodies do not stain T cells stimulated with one signal (either PMA or ionomycin) in a detection method carried out in a conventional way. 8F4-specific antibodies produce a typical staining pattern of tonsillar T cells and recognize an antigen of about 55 to 60 kDa in a non-reducing SDS-PAGE and of about 27 kDa and about 29 kDa in a reducing SDS-PAGE on activated T lymphocytes.

In another embodiment, the invention relates to hybridoma cells which produce the monoclonal antibodies according to the invention.

In another embodiment, the invention relates to the use of substances which inhibit the biological activity of the polypeptide 8F4 according to the invention as pharmaceuticals. The use of the monoclonal antibodies according to the invention, natural or synthetic ligands, agonists or antagonists of the 8F4 molecule is particularly preferred. These substances can be used as pharmaceuticals for the prevention or therapy of disorders in which the immune system is involved, in particular for the treatment or autoimmune diseases or for prevention of rejection reactions in organ transplants. Blockade of the interaction of the 8F4 antigen with its receptor improves, for example, the prevention of organ rejection because such a blockade affects only previously activated T lymphocytes. Another embodiment of the invention relates to the use of the polypeptide according to the invention as pharmaceutical. The polypeptide according to the invention can be used in particular for the prevention or therapy of disorders in which the immune system is involved, in particular for the treatment of cancers, AIDS, asthmatic disorders or chronic viral diseases such as HCV or HBV infections.

The polypeptide according to the invention can likewise be introduced into cells in a conventional

way so that these cells for example constitutively ex-
press the polypeptide. For example, the nucleic acid
sequence encoding the polypeptide or a vector compri-
sing the nucleic acid sequencing encoding the polypep-
tide, for example the cDNA or genomic DNA, promoters,
enhancers and other elements required for expression of
the nucleic acid sequence can be inserted into a cell.
The 8F4 cDNA (2641 nucleotides) depicted in Fig. 16
(SEQ ID NO:1) or fragments or derivatives thereof, is
preferably employed for expression of the polypeptide
according to the invention or fragments thereof.

The polypeptide according to the invention
can also be introduced for example by means of liposo-
mes into cells which then form the polypeptide on their
cell surface. These cells can be used as pharmaceuti-
cals according to the invention, in particular for
restoring correct regulation of the human immune sy-
stem, as occurs within the framework of numerous chro-
nic infectious diseases, for example within the frame-
work of AIDS, asthmatic disorders or in chronic viral
hepatitis (for example HCV, HBV), or for stimulating
the immune system in vitro or in vivo such as, for ex-
ample, be used for the therapy of cancers.

In another embodiment, substances which spe-
cifically recognize the polypeptide according to the
invention are used for diagnosing disorders in which
the immune system is involved, the substances embracing
in particular a monoclonal antibody, natural or synthe-
tic ligands, agonists or antagonists. It is possible to
use for the diagnosis for example an ELISA detection,
flow cytometry, Western blot, radioimmunoassay, nephe-
lometry or a histochemical staining. The substances
which recognize the polypeptide according to the inven-
tion also comprise nucleic acid sequences, the latter
preferably being employed for hybridization and/or
nucleic acid (RNA, DNA) amplification (for example
PCR).

In another embodiment, the invention relates
to substances which have a positive or negative effect
on (modulate) the signal transduction pathway of the
polypeptide according to the invention into the T cell,
and to the use of these substances as pharmaceuticals.

In another embodiment, the invention relates
to substances which prevent up-regulation of the poly-
peptide according to the invention on the T-cell sur-
face, and to the use thereof as pharmaceuticals.

- 9 -

In another embodiment, the polypeptide accor-
ding to the invention or fragments thereof is expressed
by a transgenic animal.

In another embodiment, the invention embraces
5   a transgenic animal in which the gene which codes for
the polypeptide according to the invention has been
switched off ("knock-out").

~~The figures serve to illustrate the inventi-~~
~~on:~~

10   Fig. 1 shows the result of an immunoprecipi-
tation of the 8F4 antigen from activated human T cells.
(a) Sodium dodecyl sulphate polyacrylamide gel electro-
phoresis (SDS-PAGE; 12% polyacrylamide gel (PAA gel))
reducing, (b) SDS-PAGE (10% PAA gel) non-reducing. The
15   conditions for elution of the antigen from the 8F4 ma-
trix are indicated. "SDS" means sodium dodecyl sulpha-
te; "DTT" means dithiothreitol, "Mr" means molecular
weight and "kDa" means kilodalton.

Fig. 2a shows the result of a flow cytometry
20   after induction of the 8F4 antigen on CD4$^+$ T cells. The
activation time for the T cells is indicated in paren-
theses. "PMA" means phorbol myristate acetate; "PHA"
means phytohaemagglutinin; "OKT3" is a monoclonal anti-
body against CD3; "MLR" means mixed lymphocyte reac-
25   tion; "mAK 9.3" is a monoclonal antibody against CD28;
"SEB" means staphylococcal enterotoxin B.

Fig. 2b shows the result for the kinetics of
induction of the 8F4 antigen on CD4$^+$ T cells after acti-
vation with PMA and ionomycin in a flow cytometry. The
30   immunofluorescence (log) is plotted against the cell
count.

Fig. 3 shows the result of a flow cytometry
for identifying molecules which are involved in the in-
duction of 8F4 in the mixed lymphocyte reaction. "bio"
35   means biotinylated antibody.

Fig. 4 shows the result of a histochemical
investigation for localization of 8F4-positive cells in
the tonsil.

Fig. 5 shows the result of an expression ana-
40   lysis of 8F4 on T and B cells from human tonsils in a
flow cytometry. "bioPE" means biotinylated antibody and
streptavidin-phycoerythrin secondary reagent.

Fig. 6 shows the coexpression of the 8F4 mo-
lecule with other activation markers (CD69, CD45) in a
45   flow cytometry.

Fig. 7 shows diagrammatically the enhanced expression of activation molecules on T lymphocytes after costimulation by 8F4. Open circles (O) represent 8F4 antibodies; triangles (♦) represent nonspecific antibodies of the same isotype; filled circles (●) represent anti-CD28 antibodies-9.3.

Fig. 8 shows a diagrammatic comparison of the costimulating effect of 8F4 with the costimulating effect of CD28. "mAk" means monoclonal antibodies; "ATAC" means "activation induced T-cell-derived and chemokine-related"; "cpm" means radioactive disintegrations per minute.

Fig. 9 shows diagrammatically the enhancement of the synthesis of the antibodies of the IgM and IgG types by B cells after costimulation of T cells. "ng" means nanogram; "ml" means millilitre; "mAk" means monoclonal antibody.

Fig. 10 shows diagrammatically the prevention of the activation-induced apoptosis of peripheral T cells after costimulation by 8F4.

Fig. 11 shows expression of the 8F4 antigen on the MOLT-4V cell line. MOLT-4V cells were stained with a fluorescein-labelled 8F4 antibody (8F4-FITC) and investigated in flow cytometry (unfilled line, comparing with an isotype control (filled line)).

Fig. 12 shows the two-dimensional gel electrophoresis. A MOLT-4V cell lysate from 300×10^6 cells was immunoprecipitated as described. The eluate was fractionated on a non-reducing SDS-PAGE (10% PAA), and the region around 60 kDa was cut out of the gel. To reduce the disulphide bridges in the 8F4 molecule, the piece of gel was incubated in 5.3 M urea, 0.5 M Tris, pH 8.0, 1% SDS, 1% ß-mercaptoethanol at 50°C for 1 h, and the free cysteine residues in the molecule were alkylated with 10 mM iodoacetamide (Sigma, Deisenhofen) (37°C, 30 min). The piece of gel was equilibrated in 1×SDS-PAGE sample buffer for a further 30 min and mounted on a 12% PAA-SDS gel (with stacking gel). After fractionation by electrophoresis, the gel underwent silver staining. The location of the 8F4 protein was determined by surface iodination (cf. Fig. 1) and is marked by a circle. (All the procedures not described in detail were carried out by standard methods, see, for example, Westermeier, R., Electrophoresis in Practice, VCH Verlagsgesellschaft, Weinheim, 1997).

Fig. 13 shows a hybridization with Oligo 1 (SEQ ID NO:3). Lambda clones immobilized on nitrocellu-

lose filters were hybridized with Oligo 1 as described in the examples. Exposure on an X-ray film is depicted (detail).

Fig. 14 shows a Northern blot analysis with the 8F4 cDNA. Hybridization of a Northern blot with the 8F4 cDNA produces a band which migrates in the gel between the 18S and 28S RNA. Fig. 14A shows the behaviour as 2-signal-dependent (see above) activation antigen: no expression in resting lymphoid cells (PBL), strong expression in PMA+ionomycin-activated CD4+ T cells and distinctly reduced expression with PMA or ionomycin alone. Fig. 14B shows the strength of mRNA expression after different stimulation times (T cells (purified via nylon wool adherence, NTC), stimulated with PMA+ionomycin). Besides this the MOLT-4 cell lines (ATCC CRL-1582) which shows only minimal expression, and on the far right the MOLT-4V which was used for the cloning and which shows a distinct signal. Also loaded is the RNA from other cell lines on which no 8F4 expression was detectable in the analysis by flow cytometry: CEM (ATCC CCL-119), HUT-102 (ATCC TIB-162), HUT-78 (ATCC TIB-161), Jurkat (ATCC TIB-152), DG75 (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ACC83), Karpas 299 (Fischer, P. et al. (1988), Blood, 72:234-240), DEL (Barbey, S. et al. (1990), Int. J. Cancer, 45:546-553).

Fig. 15 shows the amino acid sequence of the polypeptide 8F4 (SEQ ID NO:2).

Fig. 16 shows the 8F4 cDNA (SEQ ID NO:1).

The following examples illustrate the invention and are not to be understood restrictively.

Example 1: Generation of the 8F4 antibody

Balb/c mice were immunized with human T cells which had previously been activated for 24 h with 33 ng/ml of the phorbol ester phorbol myristate acetate (PMA) (Sigma, Deisenhofen) and with 200 ng/ml of the Ca^{2+} ionophore ionomycin (Sigma, Deisenhofen) (so-called "2-signal activation"). After boosting three times, the spleen cells of the mice were fused with the myeloma P3X63Ag8.653 (ATCC No. CRL-1580), and antibody-secreting hybridomas were generated by standard methods; cf. Peters and Baumgarten, Monoclonal Antibodies, Springer, Heidelberg, 1992. The resulting antibodies were screened for activated versus resting T cells

in flow cytometry. Activated ("2-signal activation") and resting T cells were incubated with the hybridoma supernatant and then labelled with a fluorescence-labelled secondary antibody; cf. Shapiro, Practical
5 Flow Cytometry, Wiley-Liss, New York, 1995. Only the antibodies which recognize molecules which were induced exclusively by PMA and the Ca^{2+} ionophore ionomycin on the T-cell surface, but not by one of the agents alone ("2-signal molecules"), were selected for further puri-
10 fication. The resulting antibodies were investigated in flow cytometry for similarity to or difference from known antibodies against activation molecules (cf. Table 1) on T cells. The criteria for this were, besides the abovementioned "2-signal dependence", the kinetics
15 of induction on stimulated T cells and the expression on various cell lines.

Example 2: Immunoprecipitation of the 8F4 antigen

20 Surface molecules from activated human T cells were iodinated with ^{125}I by standard methods and immuno-precipitated with the antibody 8F4 by standard methods; cf. Goding, Monoclonal Antibodies: Principle and Practice, Academic Press, London, 1996. The antibody for
25 the immunoprecipitation was coupled by the method of Schneider et al., *Journal of Biological Chemistry* 257 (1982), 10766-10769, to protein G (Pharmacia, Freiburg) (8F4 matrix). The matrix was washed as described by Schneider et al., see above. The immunoprecipitated 8F4
30 molecule was analysed for its molecular mass in an SDS-PAGE (non-reduced and reduced) in a conventional way; Goding, see above.

Example 3: Flow cytometry

The 8F4-carrying T cells were analysed in flow
35 cytometry by standard methods; cf. Shapiro, Practical Flow Cytometry, Wiley-Liss, New York, 1995.

Exemplary embodiment 3.1: Flow cytometry after induction of the 8F4 antigen on $CD4^+$ T cells.

CD4$^+$ T cells from peripheral blood were stimulated with various agents in a conventional way, and investigated for expression of the 8F4 molecule in flow cytometry by a conventional method. The activation time

5 for the T cells was between 24 hours and 144 hours with the various agents. Modes of activation: phorbol myristate acetate (PMA; 33 ng/ml), ionomycin (200 ng/ml), phytohaemagglutinin (PHA 1.5 mg/ml), OKT3 (monoclonal antibody against CD3), mixed lymphocyte reaction (MLR,

10 between 50,000 CD4$^+$ T cells and 100,000 B cells), mAk 9.3 (monoclonal antibody against CD28), staphylococcal enterotoxin B (SEB, 0.1 ng/ml). Analysis revealed that various stimuli are suitable for inducing the 8F4 molecule on T cells, but the expression density differs.

15 The most potent stimuli, besides the highly active pharmacological agents PMA and ionomycin, are those which represent a costimulatory situation such as, for example, accessory cells in the MLR or the costimulating mAk 9.3.

20 Exemplary embodiment 3.2: Kinetics of induction of the 8F4 antigen on CD4$^+$ T cells after activation with PMA and ionomycin.

CD4$^+$ T cells from peripheral blood were stimulated with PMA (33 ng/ml) and ionomycin (200 ng/ml) in

25 a conventional way and investigated after 0, 4, 8, 12, 24 and 48 hours for expression of the 8F4 molecule by flow cytometry in a conventional way. The molecule is detectable on the surface after only four hours, and thus belongs to the class of relatively early activa-

30 tion antigens. There is still good expression of the antigen even after 48 hours.

Exemplary embodiment 3.3: Flow cytometry to identify molecules which are involved in the induction of 8F4 in the "mixed lymphocyte reaction".

50,000 CD4$^+$ T cells from peripheral blood were cocultivated with 100,000 allogeneic tonsillar B cells for 6 days (37°C, 5.2% CO_2, 200 μl of RPMI 1640 with 10% FCS in 96-well round-bottom plates) and then investi-
5   gated for expression of the 8F4 molecule in flow cytometry. At the start of cultivation, various antibodies (anti-CD80, anti-CD86, anti-MHCII; all 10 mg/ml) were added to the culture in order to examine the dependence of 8F4 induction on these molecules. Expres-
10 sion of 8F4 can be blocked only by blockade of the CD86/CD28 interaction, but not by blockade of CD80. The blockade effect in this case is even stronger than the blockade of MHCII (positive control).

Exemplary embodiment 3.4: Expression of 8F4 on T and B
15 cells from human tonsils.

B cells and T cells from tonsillar tissue from various sources were purified in a conventional way and investigated by flow cytometry for expression of the 8F4 molecule. Whereas the signal was not unambiguously sig-
20 nificant on B cells, there was expression of the 8F4 molecule in varying density by about 50-80% of tonsillar T cells. It is possible in this case to identify two populations differing in the level of fluorescence (8F4 high and low, respectively), and differing in ex-
25 pression on the various tonsils. Thus, for example, tonsils show a pronounced 8F4 low population and other tonsils show a pronounced 8F4 high population.

Exemplary embodiment 3.5: Coexpression of the 8F4 molecule with other activation markers.

30 T cells purified from human tonsils were analysed in 2-colour flow cytometry for coexpression of the 8F4 molecule with other activation markers. In tonsils, 8F4 is coexpressed with CD69 as well as with variants of the CD45 molecule. In this case, the 8F4 high cells are
35 unambiguously correlated with a CD45R0 expression,

while the 8F4-negative cells carry the phenotype CD45RA. CD45RA is mainly expressed by so-called "naïve" T cells, whereas CD45RO is associated with an effector cell function. The 8F4$^+$ cells are thus mainly "mature" T

5 cells. CD45RO and CD45RA are isoforms of CD45.

Example 4: Localization of 8F4-positive cells in the tonsil

Tonsillar tissue in frozen sections was stained with the 8F4 antibody in the APAAP technique (alkaline

10 phosphatase-anti-alkaline phosphatase) by standard methods. 8F4$^+$ cells were found preferentially in the germinal centre of the tonsils, but also in part in the T-cell zone of the tonsils.

Example 5: Costimulation of T lymphocytes

15 96-well plates were coated with a goat anti-mouse Ig antibody (20 μg/ml), washed, and loaded with the anti-CD3 monoclonal antibody OKT3 (various dilutions of an ascites) and the 8F4 antibody according to the invention (2 μg/ml). The OKM1 antibody or the 2A11

20 antibody (both 2 μg/ml) were used as isotype control.

Exemplary embodiment 5.1: Enhanced expression of activation molecules on T lymphocytes after costimulation by 8F4.

Purified CD4$^+$ T cells from peripheral blood were

25 activated with various concentrations of the monoclonal antibody OKT3 and, at the same time, costimulated with the 8F4 antibody or a nonspecific antibody of the same isotype. As comparison, costimulation was carried out with the anti-CD28 antibody-9.3, one of the strongest

30 known costimulatory antibodies. Even with optimal stimulation by CD3, a costimulatory effect is still to be seen both with the mAk 8F4 and with the mAk 9.3. In the suboptimal OKT3 region, that is to say the region in which complete T-cell activation cannot be achieved

without costimulation, both antibodies are able to in-
crease the expression of other activation antigens by a
factor of 4 to 100, and the effect of the anti-CD28 an-
tibody is still visible even at very high OKT3 dilu-
tions. This is attributable to the fact that with very
weak OKT3 stimulation the 8F4 antigen is no longer
brought to the cell surface and thus cannot be
crosslinked by the mAk 8F4 either.

Exemplary embodiment 5.2: Comparison of the costimulat-
ing effect of 8F4 with the costimulating effect of
CD28.

Purified CD8$^+$ T cells were stimulated with a
suboptimal concentration of the monoclonal antibody
OKT3 for 51 h. The costimulators employed were antibody
8F4, antibody 9.3 (anti-CD28) and isotype controls
(2 μg/ml each). After completion of the stimulation
time, the T-cell proliferation rate was determined by
^{3}H-thymidine incorporation. In parallel cultures, the
supernatant was removed and the concentration of the
cytokines ATAC/lymphotactin and IL-2 was determined.
8F4 and CD28 differ greatly from one another in rela-
tion to IL-2 synthesis. CD28 costimulation leads, as
also described in the prior art (Chambers and Allison,
Current Opinion in Immunology 9 (1997), 396-404), to
very extensive IL-2 secretion. By contrast, IL-2 pro-
duction with 8F4 is below the detection limit. However,
proliferation is comparable in the two mixtures, and
thus the autocrine growth of the T cells must be at-
tributed to other factors on costimulation of 8F4. The
two antibodies also differ scarcely at all in the
costimulatory effect in relation to secretion of the
lymphokine ATAC.

Example 6: Determination of the immunoglobulins synthe-
sized by B cells after interaction with 8F4-
costimulated T cells

96-well plates were coated with a goat anti-mouse Ig antibody (20 μg/ml), and loaded with the anti-CD3 monoclonal antibody OKT 3 (1:500 to 1:80,000 ascites) and the 8F4 antibody according to the invention
5 (2 μg/ml). The OKM1 antibody or the 2A11 antibody was used as isotype control. In some experiments, a costimulation was carried out with a CD28-specific antibody ("9.3") for comparison; cf. Hara et al., *Journal of Experimental Medicine* 161 (1985), 1513-1524. 50,000
10 purified (Magnetobeads, Dynal, Hamburg) CD4$^+$ T cells (>95% purity) from peripheral blood and 25,000 allogenic tonsillar B cells (negative selection by T-cell rosetting with sheep erythrocytes, 96% purity) were pipetted into each well of the culture plates pretreated
15 in this way, and cocultivated for 8 days. After this period, the supernatant was removed and analysed for the concentration of secrete immunoglobulins of the IgM and IgG types in an ELISA in a conventional way; cf. Nishioka and Lipsky, *Journal of Immunology* 153 (1994),
20 1027-1036.

Exemplary embodiment 6.1: Enhancement of the synthesis of antibodies of the IgM and IgG types by the B cells after costimulation of T cells.

Purified CD4$^+$ T cells from peripheral blood were
25 cocultivated with allogeneic B cells from tonsils for 8 days in a conventional way. With suboptimal stimulation of the T cells with the OKT3 antibody, the costimulation of the T cells by 8F4 enhances the secretion of IgM and IgG immunoglobulins by a factor of 40.

30 Example 7: Prevention of the activation-induced apoptosis of peripheral T cells after costimulation by 8F4.

Peripheral T cells (purified by nylon wool adherence in a conventional way), were stimulated with PHA (1.5 mg/ml) for 20 h and cultivated with IL-2 for
35 6 days. The cells were then restimulated by OKT3 with

and without costimulation by mAk 8F4 (2 μg/ml). The apoptosis was determined by staining the DNA with propidium iodide in flow cytometry (FACS). With suboptimal stimulation via the T-cell receptor complex, costimulation by 8F4 can reduce the proportion of apoptotic cells by a factor of 4.

Example 8: Cloning of the cDNA coding for the 8F4 protein

A cell line (MOLT-4V) which expresses the 8F4 antigen constitutively was identified in flow cytometry by staining with a fluorescent dye-coupled 8F4 antibody (Fig. 11). The MOLT-4V line is a variant of the human T-cell line MOLT-4 (American Type Culture Collection (ATCC) CRL-1582).

This cell line was used for preparative purification of the 8F4 antigen with the aid of the monoclonal antibody:

The cells were cultivated on a large scale (150 l) in roller culture bottles and removed by centrifugation, and the cellular proteins were extracted using a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF (Sigma, Deisenhofen), 1% NP-40 (Boehringer, Mannheim)). Cell nuclei and other insoluble constituents were removed by ultracentrifugation. The cell lysate obtained in this way was preincubated with Sepharose CL4-B (Pharmacia, Freiburg) for 2 h in order to remove proteins which bind nonspecifically to Sepharose. Incubation then took place with the 8F4 immunoaffinity matrix described in Example 2 above (4 h at 4°C). The matrix was packed into a column and then washed several times under conditions with which there is exclusive removal of nonspecifically binding proteins (1.50 mM Tris, pH 8.0, 300 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40; 2.50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40, 0.1% SDS; 3.

0.2 M glycine pH 4.0, 0.5% CHAPS (Merck, Darmstadt)). The 8F4 antigen was eluted from the matrix with 0.2 M glycine, pH 2.5, 0.5% CHAPS. The eluate was concentrated by ultrafiltration (Amicon Centricon 10, Milli-
5   pore, Eschborn).

In order to achieve further purification of the 8F4 molecule, the dimeric structure of the molecule (see Fig. 1) was utilized in a two-dimensional gel electrophoresis (nonreducing/reducing): since most pro-
10   teins occur as monomer, they migrate on a diagonal in gel electrophoresis, whereas the 8F4 molecule migrates at 55-60 kDa in the 1st dimension (nonreducing) and at 27 and 29 kDa (Fig. 12) in the 2nd dimension (reducing).

15   For preparative fractionation, the immunopre-cipitates from in each case 20×10^9 cells were prepared as described above for Fig. 12 and fractionated in two-dimensional gel electrophoresis, the gel was stained with Coomassie blue G250 (Biorad, Munich) and the areas
20   indicated in Fig. 12 were separately cut out of the gel (8F4-27 kDa and 8F4-29 kDa respectively).

For peptide microsequencing, the proteins from in each case 4 pieces of gel were digested with trypsin and eluted from the gel. The tryptic fragments were
25   fractionated by HPLC and individual fractions were sub-jected to Edman degradation (method described in detail in Groettrup, M. et al. (1996), Eur. J. Immunol., 26:863-869).

Sequencing of the 8F4-29 kDa sample revealed, besides
30   fragments of known proteins, a peptide sequence XRLTDVT for which no human correlate was found in any of the protein databases.

Unambiguous translation back of a protein sequence into a DNA sequence is not possible. Thus,

translation of the above peptide sequence back into an oligonucleotide with 17 nucleotides results in 2048 permutations. However, a specific method (Wozney, J.M. (1990), Methods Enzymol. 182:738-751) makes it possible to screen a cDNA bank with degenate oligonucleotides. On the basis of the peptide sequence found, 2 oligonucleotides (Oligo 1 (SEQ ID NO:3); MGN CTS ACN GAY GTN AC, 512 permutations; Oligo 2 (SEQ ID NO:4): MGN YTD ACN GAY GTN AC, 1024 permutations) were synthesized.

For screening, a cDNA bank was constructed from the MOLT-4V cell line also used for the protein purification:

Complete RNA was isolated by the guanidinium/CsCl method (Chirgwin, J.M. et al. (1979), Biochemistry 18:5294-5299), and mRNA was concentrated on Oligo-dT-cellulose columns (Gibco BRL, Eggenstein). Synthesis of the first and second cDNA strands was carried out using a commercial cDNA synthesis system (Gibco BRL, Eggenstein) using Oligo-dT primers in accordance with the manufacturer's instructions. The cDNA was ligated via EcoRI adaptors into the Lambda ZAPII vector (Stratagene, Heidelberg).

The cDNA bank was plated out by standard methods (Vogeli, G. and Kaytes, P.S. (1987), Methods Enzymol., 152:407-515) and the Lambda DNA was immobilized on nitrocellulose filters (Optitran BA-S 85, Schleicher & Schuell, Dassel).

The abovementioned oligonucleotides were radio-labelled using T4 polynucleotide kinase (NEBL, Schwalbach) and γ-^{32}P ATP (NEN Du Pont, Brussels) (Wallace, R.B. and Miyada, C.G. (1987), Methods Enzymol., 152:432-442).

Hybridization of the filters took place in a buffer described for degenerate oligonucleotides (Wozney, J.M. (1990), Methods Enzymol. 182:738-751) with 3 M tetramethylammonium chloride (Roth, Karlsruhe) at 48°C. The filters were washed as described in the abovementioned reference, the washing temperature being 50°C. Exposure of these filters on an X-ray film revealed about 50 positive clones per 100,000 plated phages (Fig. 13).

6 clones were further characterized by transferring them by in vivo excision, using the method described by the manufacturer of the vector (Stratagene, Heidelberg), into a plasmid vector, and partially sequencing with T3 and T7 primers (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, USA). One of the clones contained a sequence which on translation provided exactly the peptide sequence which was sought. This clone was used for hybridization of a Northern blot (Fig. 14) (Kroczek, R.A. (1993), J. Chromatogr., 618, 133-145). The expression pattern of the mRNA corresponded exactly to the expression of the 8F4 molecule as was known from investigations on the monoclonal antibody by flow cytometry. Since the clone which was found contained only the 3' end of the cDNA sought, a fragment on the 5' side was used to isolate the complete 8F4 cDNA. Several clones were sequenced on both strands.

The 8F4 cDNA (2641 nucleotides) is depicted in Fig. 16 and in the sequence listing under SEQ ID NO:1, and codes for a protein having 199 amino acids (Nucleotides 68-664), depicted in Fig. 15 and in the sequence listing under SEQ ID NO:2. Sequencing of several independent clones from the cDNA bank showed some deviations from the sequence shown here, but these are all in the 3'-untranslated region:

Pos. 909-910:deletion

Pos. 1631:T->C

Pos. 2074:G->T

Pos. 2440:G->C

5   Pos. 2633: alternative polyadenylation site

Table 1:

Table 1 summarizes the antibodies used (clone), their source of origin (source), the specificity for their particular antigen (specificity) and, where appropriate, their labelling (label).

Speci-ficity	Label	Isotype	Clone	Source
CD3	Cy-Chrome	IgG1	UCHT1	Pharmingen, Hamburg
CD3	-	IgG2a	OKT3	ATCC CRL-8001
CD11b	-	IgG2b	OKM1	ATCC CRL-8026
CD25	FITC	IgG2a	B1.49.9	Immunotech, Hamburg
CD28	-	IgG2a	9.3	Immunex Corp., Seattle
CD45RA	Cy-Chrome	IgG2b	HI100	Pharmingen, Hamburg
CD45RO	FITC	IgG2a	UCHL1	Immunotech, Hamburg
CD69	FITC	IgG1	FN50	Pharmingen, Hamburg
CD80	-	IgG1	L307.4	Becton Dickinson, Heidelberg
CD86	-	IgG2b	IT2.2	Pharmingen, Hamburg
CD154	FITC	IgG1	TRAP-1	Hybridoma[1]
MHCII	-	IgG2a	L243	ATCC HB-55
8F4	-	IgG1	8F4	Hybridoma[1]
8F4	Biotin	IgG1	8F4	Hybridoma[1]
Isotype IgG1	-	IgG1	2A11	Hybridoma[1,2]
Isotype IgG1	FITC	IgG1	2A11	Hybridoma[1,2]
Isotype IgG1	Biotin	IgG1	ASA-1	Hybridoma[1]

[1]   The hybridoma cell line was generated in a conventional way, and the antibody was purified and labelled where appropriate.

[2]   Directed against a synthetic peptide

The antisera and secondary reagents used in the examples were purchased from: goat anti-mouse Ig, FITC conjugated, from Jackson Immuno Research Lab., USA; Streptavidin, PE-conjugated, from Jackson Immuno Research Lab., USA; rabbit anti-mouse Ig fraction, from Sigma, Deisenhofen.

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

Priority applications DE 19741929.1 filed September 23, 1997 and DE 19821060.4 filed May 11, 1998 including the specification, drawings, claims and abstract, are hereby incorporated by reference. All publications cited herein are incorporated in their entireties by reference.